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CONTRACTING ORGANIZATION:
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14. ABSTRACT Metastasis to distant vital organs (bone, lung, brain) is the most devastating feature of breast cancer. We proposed to extend our current integrative genomic, proteomic and transcriptomic analysis on the crosstalk between breast cancer cells and bone and lung microenvironments during organ-tropic metastasis. An understanding of secreted metastasis regulators (extracellular proteins, cell-free nucleic acids and small vesicles –exosomes-) has tremendous potential to improve the diagnosis, prognosis and treatment of breast cancer. We hypothesized that tumor and stromal cells communicate via secreted and exosomal proteins and miRNAs to promote organotropic metastasis. Therapeutic disruptions of these communication pathways may significantly increase diagnostic options, improve treatment efficacy and survival of breast cancer patients. The **objectives** of our proposal are to comprehensively analyze secreted and exosomal proteins and miRNAs that are regulators of bone and lung metastasis, to characterize their function in mediating tumor-stroma interactions, and to determine the potential of utilizing such circulating factors as biomarkers and therapeutic targets. Our specific aims are: 1) Identification and functional characterization of secreted factors promoting bone and lung metastasis; 2) Determination of the role of exosomes in metastatic progression and niche formation; 3) Clinical analysis of metastatic secretome and exosomes.

15. SUBJECT TERMS
breast cancer, exosomes, organ-specific metastasis

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DOD Grant/Contract (Award Number W81XWH-13-1-0427)

YEAR 3 RESEARCH REPORT

Grant Title: Organ-tropic metastatic secretomes and exosomes in breast cancer

INTRODUCTION:

Background: Over 90% of breast cancer deaths are caused by the metastatic spread of tumors to vital secondary organs, including bone and lung. Pathogenesis of metastasis is likely mediated by intercellular communication between tumor cells and the stromal microenvironment. In addition to direct cell-cell contact, many of such tumor-stromal interactions occur via secreted factors, such as growth factors, cytokines, cell-free nucleic acids and small vesicles called exosomes. A comprehensive understanding of secreted molecular mediators of tumor-stroma interactions in organ-tropic metastasis of breast cancer to bone and lung has tremendous potential impact on improving the diagnosis, prognosis and treatment of breast cancer. We postulated that tumor and stromal cells communicate via secreted and exosomal proteins and miRNAs to promote organotropic metastasis. Therapeutic disruptions of these pathways may significantly improve disease diagnosis and prognosis, as well as reducing the morbidity and mortality associated with metastasis. Recently, collaborations between the Lyden and Kang laboratories have demonstrated that exosomes are one of the tumour-derived factors inducing vascular leakiness, inflammation, and BM progenitor cell recruitment during pre-metastatic niche formation and metastasis (Peinado *et al*, Nature Medicine, 2012). The functional biomolecules (*i.e.*, proteins, lipids, RNAs, DNA) contained by exosomes can be horizontally transferred to recipient cells. We showed that an “exosomal protein signature” could identify melanoma patients at risk for metastasis to non-specific distant sites. Moreover, in the context of pancreatic cancer exosomes, we defined the sequential steps involved in liver pre-metastatic niche induction (Costa-Silva *et al*, Nature Cell Biology, 2015). Importantly, we demonstrated that integrins present on tumor exosomes determine organotropic metastasis (Hoshino *et al*, Nature, 2015). The **objectives** of our proposal are to comprehensively identify secreted and exosomal proteins and miRNAs that are functional mediators of bone and lung metastasis, to characterize their functional mechanisms in mediating tumor-stromal interactions, and to determine the potential of utilizing such circulating factors as biomarkers and therapeutic targets.

Summary of the tasks/aims proposed and achievements:

Task 1: Identification and functional characterization of secreted factors promoting bone and lung metastasis (Months 1-48).
Given the paucity of studies on secreted proteins and miRNAs with functional relevance in metastatic organ-tropism we are currently analyzing secretomes and

extracellular miRNAs from lung (**Lyden laboratory**) and bone metastatic breast cancer cells (**Kang Laboratory)**.

Task 1a: Identify differentially secreted miRNAs associated with bone-tropism of breast cancer cells **(Months 1-48)**. **Dr. Kang's group is responsible for this task.**

Task 1b: Identify differentially secreted proteins and miRNAs associated with lung-tropism of breast cancer cells **(Months 1-48)**.

- RNA isolation from lung metastatic cancer cells has been optimized and we are currently performing RNA-Seq at the WCMC Genomics Facility. The data is being deconvoluted and undergoing bioinformatic analysis at the WCMC Institute for Computational Biology, under the supervision of Dr. Olivier Elemento. We used the parental MDA-MB-231 as well as sublines with high and low lung metastatic tropism and the MCFCA1h (poorly metastatic) and MCFCA1a (highly metastatic) pair of human breast cancer cell lines. We also plan to isolate miRNAs from the 4T1 series of mouse mammary tumor cell lines with progressively higher lung metastatic abilities and are planning on sequencing these **(Months 1-6 of year 4)**.
- Once top lung tropic secretome miRNA and proteins are identified, we plan to test the detection of secretome candidate proteins/miRNAs in animal models: healthy, primary tumor bearing, spontaneous and experimental lung metastasis. 40 nude and Balb/c mice will be used **(Months 6-12 of year 4, initial ACURO approval was obtained, but the ACURO is currently under re-review following the 3 year renewal of the Lyden IACUC protocol 0709-666A, approved 10/20/2016)**.
- We are currently working on methods to optimize the comparison between secreted and exosomal miRNA/proteins isolated from lung-tropic and parental control cell lines.

Outcome and Milestones: We have identified distinct protein profiles **(Lyden**) of breast cancer cell lines with differential lung metastatic capabilities whose pathological relevance can be validated in animal models of lung metastasis. We have published a subset of these the results, pertaining to lung exosome protein content, specifically integrins, at the beginning of the thrid year of funding, ahead of the milestone timetable. For **Year 4** of funding studies, we will focus on moving further the molecular and functional analysis of lung and brain tropic exosomal proteins and miRNA, and their effects on stromal cells constituting pre-metastatic niches in these organs **(Lyden**).

Task 1c: Stroma-derived miRNAs as biomarkers and potential therapeutic targets **(Months 1-24). Dr. Kang's group is responsible for this task.**

Task 2: Determination of the role of exosomes in metastatic progression and niche formation (Months 1-48). ***Task will be performed by Dr. Kang (bone metastatic exosomes & functional analysis), Dr. Lyden (lung metastatic exosomes & functional analysis), Dr. Garcia (proteomics), Sequencing Core Facilities (RNA-Seq).***

Based on our previous studies, tumor-derived exosomes can promote metastasis by transfer of functional factors. This aim will analyze exosomal proteins and miRNAs released from lung metastatic breast cancer cells to identify metastasis regulators.

Task 2a: Identify differences in exosomal protein/miRNA composition between highly and poorly bone metastatic breast cancer cells and determine the function of the candidate exosomal bone metastasis regulators **(Months 1-48)**. **Dr. Kang's group is responsible for this task.**

Task 2b: Identify differences in exosomal protein/miRNA composition between highly and poorly lung metastatic breast cancer cells and determine the function of the candidate exosomal lung metastasis regulators **(Months 1-48)**.

• For the third year of funding we have focused on further mining our comprehensive mass spectrometry proteomic database of exosomes isolated from various organotropic cell lines, focusing specifically on integrins shared by metastatic cells. We isolated and characterize exosomes from cancer cells with different metastatic capabilities, and analyzed protein by mass-spectrometry. We used the parental MDA-MB-231 as well as sublines with high and metastatic potential. **(Months 1-6 of year 4)**.

We postulated that exosomal adhesion molecules could regulate local microenvironments within future metastatic organs. Quantitative mass spectrometry of brain-, lung-, and liver-tropic metastatic exosomes identified integrin beta 1 ($ITG\beta_1$) among the top 40 most abundant adhesion molecules. Moreover, $ITG\beta_1$ was present in all metastatic cell lines, but not in non-metastatic and normal breast fibroblast cell lines such as WI-38 (Table 1 and 2). These data indicate a correlation between exosomal $ITG\beta_1$ and metastatic potential (Fig.1). Last but not least, $ITG\beta_1$ can also partner with $ITG\alpha_6$ and may be an additional mediator of lung metastasis.

Therefore, we focused on examining the functional role of exosomal $ITG\beta_1$ (and its extracellular matrix binding partner fibronectin) levels in exosomes isolated from the MDA-MB-231 breast cancer cell line and its lung tropic variants (MDA-MB-231-4175). We first generated the tools to examine the functional role of exosomal $ITG\beta_1$ in metastasis. We employed both shRNA and CRISPR technology, and successfuly knocked down/ablated $ITG\beta_1$ expression in MDA-MB-231 cells and thus in the exosomes they secrete (Figure 1A). We then characterized the *in vitro* growth of these $ITG\beta_1$-KD cells, as well as their responses to drugs such as MAPK inhibitors, and found these properties to be somewhat affected by $ITG\beta_1$ loss of function, consistent with previous reports (Figure 1B, C). To begin to address the functional role of exosomal $ITG\beta_1$ in metastasis, we assesed the capacity of $ITG\beta_1$-KD and control exosomes to conditon lung metastatic niches in naïve animals. We found that $ITG\beta_1$ exosomes have a markedly diminished capacity to home and be uptaken in the lung microenvironment *in vivo*, as evidenced by whole mount lung near-infrared imaging (Figure 2).

Table 1. Integrin expression in human metastatic cell line-derived exosomes, based on qualitative mass spectrometry analysis.

Sites of metastasis	None		Bone	Brain				Lung									Liver									
Cell type	Lung Fibroblast	Mammary Epithelial	Breast Cancer			Melanoma		Breast Cancer			Osteosarcoma	Rhabdomyosarcoma		Wilms' Tumor		Melanoma	Uveal melanoma	Colorectal Cancer			Pancreatic Cancer				Gastric Cancer	
Cell line	WI-38	MCF10A	1833	231	231BR	1205Lu	SBcl2	4T1	4T07	4TO8	143B	RD	CTR	G401 WT1	CCL51	1205Lu	Primary culture	HCT116	HT29	SW620	BXPC-3	HPAF-II	MiaPaCa-2	PANC-1	SNU1	SNU16
$ITGA_1$							+	+		+							+			+	+					
$ITGA_2$			+	+	+	+	+	+	+	+	+			+		+	+	+	+	+	+	+	+			
$ITGA_{2b}$							+														+					
$ITGA_3$		+	+	+	+			+	+	+	+						+	+	+		+	+	+			
$ITGA_4$						+					+	+		+	+	+	+									
$ITGA_5$							+	+	+	+		+	+		+								+	+	+	
$ITGA_6$				+	+		+	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+	+	+	+
$ITGA_{11}$										+																
$ITGA_v$				+		+	+	+	+	+	+	+	+		+	+	+	+		+	+	+	+	+	+	
$ITGB_1$		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
$ITGB_3$				+	+	+	+	+		+	+	+	+	+	+	+	+	+		+	+	+	+			
$ITGB_4$							+	+	+	+								+	+	+	+	+	+			+
$ITGA_L$	+			+			+	+	+	+		+	+		+		+	+	+	+	+	+	+	+	+	+
$ITGA_E$																					+	+	+			

Table 2. Integrin expression in human versus murine metastatic cell line-derived exosomes, based on qualitative mass spectrometry analysis.

Human

Sites of metastasis	Majority to lung	Lung and liver
Cell type	Breast cancer	
Cell line	MDA-MB-231	MDA-MB-468
$ITGA_1$		
$ITGA_2$	+	+
$ITGA_{2b}$		
$ITGA_3$	+	+
$ITGA_4$		
$ITGA_5$		+
$ITGA_6$	+	+
$ITGA_v$		+
$ITGB_1$	+	+
$ITGB_3$	+	+
$ITGB_4$		+
$ITGB_5$		+
$ITGB_6$		+

Murine

Sites of metastasis	Lung	Liver
Cell type	Breast cancer	Pancreatic cancer
Cell line	E0771	Pan02
$ITGA_1$		
$ITGA_2$		
$ITGA_{2b}$		+
$ITGA_3$	+	+
$ITGA_4$		
$ITGA_5$	+	+
$ITGA_6$	+	+
$ITGA_v$	+	+
$ITGB_1$	+	+
$ITGB_3$	+	+
$ITGB_4$		+
$ITGB_5$		+
$ITGB_6$		

Figure 1. Characterization of ITGβ$_1$-KD exosomes. A) Western blot analysis of cell lysates and exosomes isolated from MDA-231 control and ITGβ$_1$-KD cells. B) Proliferation and C) Morphology and response to MAPK inhibition in MDA-231 control and ITGβ$_1$-KD cells.

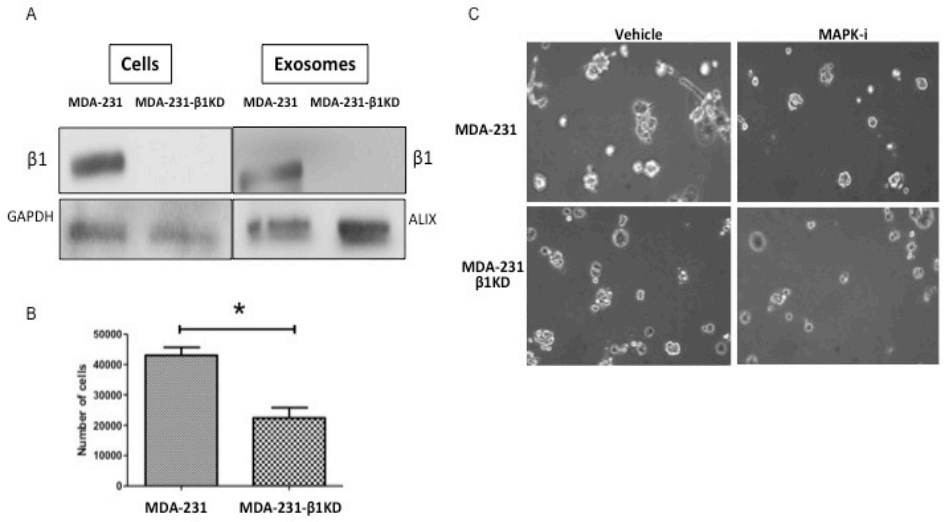
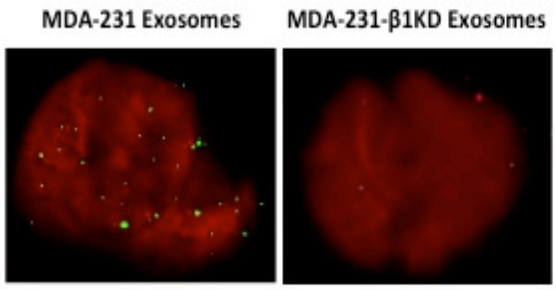


Figure 2. ITGβ$_1$-KD exosomes fail to home and to be uptaken in the lung microenvironment. Naïve nude mice were injected retro-orbitally with MDA-231 control and ITGβ$_1$-KD exosomes labeled with near-infrared lipophilic dyes, sacrificed 24 hours later and the lungs were imaged using the Odyssey system.



We then addressed the functional role of exosomal ITGβ$_1$ in lung metastasis, by assessing the capacity of ITGβ$_1$-KD and control exosomes to conditon lung metastatic niches in naïve animals, and to influence subsequent homing of tumor cells to these niches. We found that ITGβ$_1$-KD exosomes have a markedly diminished capacity condition the lung microenvironment for metastasis, as evidenced by the significant reduction in tumor cell homing to lung compared to

niches conditioned by ITGβ_1-expressing exosomes (Figure 3). We are currently focusing our attention on determining the mechanisms through which exosomal ITGβ_1 conditions metastatic niches. Moreover, since ITGβ_1 is one of the few integrins expressed in exosomes isolated from bone-tropic BC cells, we aim to ablate its expression in bone-tropic cell lines and their respective exosomes an investigate its function in preparing bone marrow niches for metastasis.

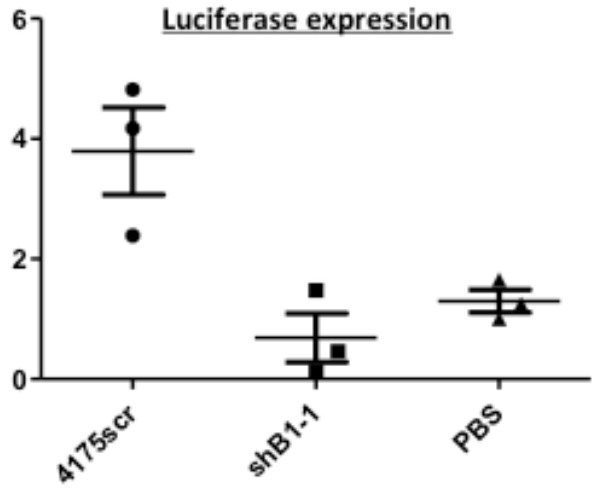


Figure 3. Quantification of metastasis upon ITGB1 knock-down in exosomes. Mice were conditioned every other day for 3 weeks with 10 micrograms of exosomes isolated from MDA-231-4175 lung tropic breast cancer cells transduced with scrambled shRNA (scr) or shRNA targeting integrin beta 1 (shB1). PBS was used as control. Mice were then injected intravenously with parental MDA-231 breast cancer cells and homing to the lung was assessed by qPCR for luciferase expression.

Future directions for year 4:

• We plan to test the detection of exosomal proteins/miRNAs in plasma of animal models: healthy, primary tumor bearing, and bone metastases bearing mice **(Months 36-48, as soon as ACURO re-approval is issued)**.

• We will further evaluate KD/OE lines of exosomal integrins and other candidates and evaluate their capabilities to potentiate metastasis or to direct organotropic metastasis. We will use exosomes from generated cell lines to treat mice bearing primary tumors and bone metastases, and evaluate their effects **(Months 36-48)**.

• We will label exosomes with dyes or epitope tags and investigate the cell identity of target cells. We will then characterize the downstream effects induced *in vivo* in target cells following exosome uptake **(Months 36-48)**.

Task 2c: Investigate the functional role of stroma-derived exosomes in metastasis **(Months 36-48)**. **Dr. Kang's group is responsible for this task.**

Outcome and Milestones: We have identified several candidate exosomal proteins and miRNAs from tumor and associated stromal cells whose manipulation changes the metastatic abilities of breast cancer cells. We expect to identify target cells and downstream effects of exosome uptake. Multiple papers reporting the role of exosomes in bone and lung metastasis will be published in years 4 and 5.

Upon receipt of ACURO re-approval for animal studies, we will focus on a detailed analysis of the *in vivo* biodistribution of exosomes isolated from organ-tropic cell lines. We will test the functional requirement for various integrins for exosome homing and uptake by ablating or overexpressing these integrins in breast cancer cell line exosomes. Moreover, we will combine exosome labeling with immunofluorescence studies using cell-type specific markers to identify the specific cells uptaking tumor exosomes in each destination organ. We will use flow activated cell sorting to isolate the cells uptaking the exosomes and perform transcriptomic and proteomic analyses to identify the changes induced by exosomes in these target cells. We will perform these approaches *in vivo* during the fourth year of funding.

Task 3: Clinical analysis of metastatic secretome and exosomes (Months 36-60). In collaboration with Dr. Bromberg (clinical sample collection and analyses) we have been accruing and isolating exosomes from plasma samples from breast cancer patients with metastasis to various sites (lung, brain, bone). Based on our proteomics analysis of metastatic breast cancer cell lines, we identified exosomal proteins, specifically integrins, functionally relevant in organ-tropic, specifically lung-tropic breast cancer metastasis, and tested these samples by ELISA (protein). We predicted that due to their extracellular localization, secreted factors represent superior biomarkers and therapeutic targets.

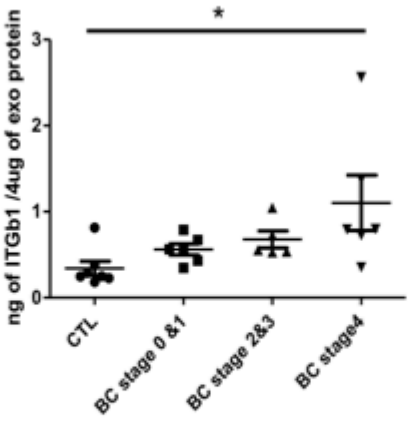


Figure 4. Levels of exosomal ITGB1 in BC patient plasma. Breast cancer patient plasma exosomal ITGB1 levels were quantified by ELISA. The amount of ITGB1 per 4 micrograms of total exosomal was analyzed in healthy control subjects (n=7), patients with breast cancer stages 0 or I (n=6), stage II and III (n=5), stage IV (n=6). Bars depict average ± s.e.m. *P<0.05.

Therefore, guided by the results of our exosomal proteomics analysis, we performed ELISA assays for plasma-derived exosomal integrins in breast cancer patients with metastasis. To determine whether exosomal $ITG\beta_1$ plasma levels can be used as a biomarker of disease burden in breast cancer patients, we have used ELISA assays to quantify the levels of exosomal $ITG\beta_1$ in the plasma of breast cancer patients at various stages of disease progression (Stage I-IV, Figure 4). We found that the levels of exosomal $ITG\beta_1$ in the plasma of breast cancer patients increase with disease progression, and could be used as a biomarker of disease progression. We are

also testing whether we can use anti-human ITGβ_1 antibodies to capture circulating exosomes from the plasma of breast cancer patients carrying this integrin, in order to enrich our patient plasma-derived exosomal preparations for tumor exosomes, thus increasing sensitivity and specificity for subsequent analyses investigating other exosomal integrins with various organ tropisms (Hoshino et al, Nature, 2015). In addition, we are interested in determining if exosomal ITGβ_1 expression can be used as an indicator for a breast cancer's propensity to metastasize to the bone. Therefore, over the following funding period, we will continue to accrue plasma from breast cancer patients, focusing on patients with bone metastasis to allow correlations of clinical data with ITGβ_1 expression.

Outcome and Milestones: We expect to validate candidates identified in Tasks 1 and 2 in a larger cohort of patient samples. We expect that some factors will have diagnostic and/or predictive value. Those with function in metastasis may become potential therapeutic targets. These results will be published in several papers in year 5.

KEY RESEARCH ACCOMPLISHMENTS:

Publications:

1) Tumour exosome integrins determine organotropic metastasis.

Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, Molina H, Kohsaka S, Di Giannatale A, Ceder S, Singh S, Williams C, Soplop N, Uryu K, Pharmer L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T, Zhang H, Hernandez J, Weiss JM, Dumont-Cole VD, Kramer K, Wexler LH, Narendran A, Schwartz GK, Healey JH, Sandstrom P, Jørgen Labori K, Kure EH, Grandgenett PM, Hollingsworth MA, de Sousa M, Kaur S, Jain M, Mallya K, Batra SK, Jarnagin WR, Brady MS, Fodstad O, Muller V, Pantel K, Minn AJ, Bissell MJ, Garcia BA, Kang Y, Rajasekhar VK, Ghajar CM, Matei I, Peinado H, Bromberg J, Lyden D. ***Nature.*** 2015 Nov 19;527(7578):329-35. doi: 10.1038/nature15756. Epub 2015 Oct 28. PMID:26524530

2) Becker A, Thakur B, Weiss JM, Kim HS, Peinado H, Lyden D. Extracellular vesicles in cancer: cell-to-cell mediators of metastasis. ***Cancer Cell, Perspective, 2016, In Press***

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Dr. Lyden has presented preliminary data from these studies in 10 national and international meetings this past year. The top meetings were:

Invited Speaker
Karolinska Institute December 2015
Stockholm, Sweden

Invited Speaker
NCI TMEN Steering Committee Meeting January 2016
Portland, Oregon

Invited Speaker
Basic &Translational Sciences Seminar Series April 2016
OHSU Knight Cancer Institute
Portland, Oregon

Invited Speaker
HemoncBMT Research Seminar April 2016
Children's Hospital Los Angeles (CHLA)
Los Angeles, California

Invited Speaker
Major Symposium Chair and Speaker April 2016
2016 AACR Annual Meeting
New Orleans, Louisiana

Invited Speaker
2016 Starr Cancer Consortium Retreat May 2016
Cold Spring Harbor, NY

Invited Speaker
International Society for Extracellular Vesicles (ISEV) May 2016
Annual Meeting 2016
Rotterdam, Netherlands

Invited Speaker June 2016
Weizmann Institute of Science
Extracellular Vesicles: Friends and Foes Conference
Rehovot, Israel

Invited Speaker September 2016
The 16th Biennial Metastasis Research Congress
Metastasis Research Society
Chengdu, China

CONCLUSION:

We have defined a specific repertoire of integrins expressed on tumour-derived exosomes, distinct from tumor cells, which dictates exosome adhesion to specific

cell-types and ECM molecules in particular organs. Furthermore, we determined that exosomal ITGβ_1 may not only function in promoting lung metastasis, but that it may be a useful biomarker of disease progression and a potential tool for enriching for breast cancer-derived exosomes from patient samples.

Therefore, in the following budget period we plan to focus on performing in vivo functional experiments to determine the role of exosomal ITGβ_1 in breast cancer metastasis to the lung and bone. Which steps in the exosome-dependent steps in the metastatic cascade does ITGβ_1 loss affect? What are the mechanisms through which exosomal ITGβ_1 mediates interaction with target cells in metastatic organs? Can ITGβ_1 be used as a biomarker for stratification of metastatic risk in patients? The identification of molecules expressed on exosomes that could "address" exosomes to specific metastatic sites could predict metastatic niches and allow foreseeing metastatic spread of tumors and metastatic organ.

We propose to test this hypothesis during next year in animal models of organ-tropic breast cancer metastasis.

Impact: Our research will unveil novel secreted and exosomal proteins and miRNAs as functional regulators of long-range communications between metastatic tumor and stromal microenvironment. Moreover, due to their extracellular localization, secreted factors represent superior biomarkers and therapeutic targets as they can readily enter body fluids where they can be non-invasively detected, targeted or restored. Thus, we expect this research to open up exciting novel avenues of clinical translation in early breast cancer and metastasis detection, prognosis and therapy. Therapeutic strategies may include, but not be limited to, monoclonal antibody for integrins, and their organ-specific downstream effectors in target cells, restoration of metastasis-inhibiting miRNAs and proteins, and modulation of signaling pathways activated by secreted proteins or targeted by miRNAs.

REFERENCES: Not applicable. No references are associated with this report.

APPENDICES: Not applicable. No appendices are attached to this report.

SUPPORTING DATA: Figures 1-4, Tables 1-2